Scanning Microscopy

Volume 1 | Number 2 Article 40

2-17-1987

Tumor Vasculature in Young and Old Hosts: Scanning Electron Microscopy of Microcorrosion Casts with Microangiography, Light Microscopy and Transmission Electron Microscopy

James G. Walmsley
University of Vermont

Scott R. Granter
University of Vermont

Miles P. Hacker
University of Vermont

Ann L. Moore
University of Vermont

William B. Ershler
University of Vermont

Scanning Microscopy, Vol. 1, No. 2, 1987 (Pages 823-830)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/87$3.00+.00

TUMOR VASCULATURE IN YOUNG AND OLD HOSTS: SCANNING ELECTRON MICROSCOPY OF MICROCORROSION CASTS WITH MICROANGIOGRAPHY, LIGHT MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY

James G. Walmsley*, Scott R. Granter,
Miles P. Hacker, Ann L. Moore, and William B. Ershler

Department of Pharmacology
University of Vermont
Burlington, VT 05401

(Received for publication July 14, 1986, and in revised form February 17, 1987)

Abstract

Tumor growth in vivo is dependent upon new blood vessel formation. When B16-F10 melanoma cells are implanted subcutaneously in young (3 mo) and old (24 mo) C57BL/6 mice the rate of growth is dependent on the age of the mice. This study involved a wide range of histological and microscopic techniques but was limited primarily to the initial phase of tumor growth. Stereological point counting from light microscopy (LM) of standard histological sections has been used to yield data regarding blood content. Tumor-bearing mice were perfused through the aorta with a fixation solution and were infused with a low-viscosity radiopaque gel (Microfil) or resin (Mercox). Soft x-rays of the whole animal were used for identifying the feeding vessels to the tumor. Tumors with Microfil were sliced and used for microangiography and light-microscopic observation while those with resin were used to make corrosion casts for scanning electron microscopy (SEM). The different characteristics of the tumor blood vessels in different aged mice were most obvious through SEM of vascular corrosion casts. In comparison with tumors in young mice those of similar size in old hosts had more necrosis, reduced presence of angiogenic features, decreased vessel density, reduced penetration into the tumor, and enhanced tortuosity of the vessel lumen. Transmission electron microscopy (TEM) revealed incompletely developed wall structure of the vessels regardless of host. The above results are consistent with the hypothesis that retarded angiogenesis may be responsible in part for the limited growth of tumors in old hosts.

Key words: Tumor Vasculature, Scanning Electron Microscopy, Light Microscopy, Transmission Electron Microscopy, Microangiography, Aging, Angiogenesis, Vascular Corrosion Casts, Vascular Pattern, Tumor Growth.

*Address for Correspondence:
Department of Biomedical Sciences
College of Medicine, University of Illinois
1601 Parkview Ave., Rockford, IL 61107 USA
Phone No. (815) 987-7730

Introduction

As age increases, the occurrence of neoplastic diseases increases in frequency (Miller, 1980; Young et al., 1976). Circumstances most likely to be responsible for this effect are failure of immune surveillance (Gatti and Good, 1970) and prolonged exposure to carcinogens (Peto et al., 1975). Younger and older patients with histologically identical tumors may display quite different clinical courses. The general condition of the host and the coexistence of other diseases would predict a more threatening clinical course for elderly patients. However, for certain tumors (Ershler et al., 1983; Saitoh et al., 1982) slower tumor growth and fewer metastases are observed. These phenomena were studied experimentally by measuring tumor growth, metastases and survival in mice of varying ages inoculated with tumors. Aged mice administered B16 melanoma had slower tumor growth, smaller final tumor volume and longer survival time (Ershler et al., 1984a) than young mice administered the same dose of the tumor. Aged mice also had fewer pulmonary metastatic colonies than young mice after an intravenous (IV) injection of tumor cells. Histological preparations suggested a more prominent fibrosis in the old mice, less vascularity and less necrosis compared with young mice (Ershler et al., 1984a). Fibrosis, as in response to surgically induced wounds or tumor growth, appears to be more pronounced in older mice (Ershler et al., 1984b). Furthermore, this fibrosis could effect angiogenesis or blood supply could alter fibrogenesis. To extend these studies the present investigation of tumor blood vessels was initiated.

The tumor volume increases slowly at first, then exponentially, before a plateau is reached. Because the characteristics of the blood vessels and vascular network are different during each of these three phases of tumor growth, this study was limited to one phase of growth, the initial one. A variety of quantitative histological procedures have been employed to provide an accurate description of the vascular architecture.

Basic techniques for the SEM of vascular corrosion casts are well established (Lametschwandtner et al., 1984). The inter-

pretation of results from vascular casts of the B16 melanoma tumors used in this study was simplified tremendously by the detailed studies of Grunt et al. (1986a; 1986b). In spite of the differences in these types of tumors the detailed anatomy of host and Lewis lung tumor vessels (Grunt et al., 1986b) are very similar to the corresponding features in the B16 melanoma in young mice. Hence, such description will only be repeated here for general comparison of old and young hosts. There have been previous studies of the vasculature of melanoma tumors in relationship to sprouting and growth but such studies did not involve SEM (Warren, 1966; Warren and Shubik, 1966). Because of the many investigations and insights of Folkman (1986) into the regulation of growth of blood vessels by hormones, the relationship of results reported here to tumor angiogenesis can be focused on structural features alone.

Materials and Methods

Mice and experimental tumor

Young C57BL/6 male mice (2-3 months old) were purchased from Charles River Laboratories, N. Wilmington, MA. Old C57BL/6 male mice were donated from the aging colonies of the National Institute on Aging maintained at Charles River Laboratories. For this strain of mice 2-3 months is considered sexually and immunologically mature (Altman and Katz, 1979) and 24 months is considered aged (Walford, 1976). The mice were housed at the animal care facility of the University of Vermont and were supplied food and water ad libitum. For each of the procedures (angiography, sizing, LM, SEM, TEM) a minimum of 5 young and 5 old animals were used. Approximately 100 tumors were observed or measured to obtain the results described.

In these studies the F10 subline of B16 murine melanoma was used. Tumor cells were stored frozen in RPMI 1640 (Flow Laboratories) with 10% V/V fetal calf serum and 7% dimethylsulfoxide. The cells were thawed and grown in minimal essential medium supplemented with 10% V/V fetal calf serum and antibiotics. The cells were detached from the flasks using Versene buffer (0.2 g/l phosphate buffered saline) and washed 3 times with the same buffer. For each experiment, cell viability was ascertained by trypan blue exclusion, and the cells were diluted to 5×10^5 viable cells/ml. For all experiments, 10^5 cells were injected subcutaneously into the right flank and animals were examined daily for survival and tumor growth. The F10 strain was chosen because preliminary studies revealed that rapid and reproducible local growth occurred after implantation in young mice. This B16 murine melanoma line has been well characterized and used for previous investigations in this laboratory (Ershler et al., 1984a, 1984b).

Mouse perfusion technique

The old or young mice bearing tumors of varying palpable sizes (100 mm^3 to 5,000 mm^3) were perfused in preparation for SEM and microangiography. Three size groups of tumors were used (palpable 100-500 mm^3, medium 1,000-1,500 mm^3, and large 2,000-5,000 mm^3). After cervical fracture the abdomen was opened along the linea alba and the descending thoracic aorta was isolated and cannulated. To dilate and clear blood vessels, the aorta was perfused at 100 mmHg with Kreb's solution containing sodium nitrite (1 mM) and heparin (10^7 USP units/ml). The fixation solution of neutral buffered isotonic formalin immediately followed the Kreb's solution. The mouse was then infused with a low viscosity radiopaque gel (Microfil) or resin (Mercox).

Histological, angiographic and microscopic techniques

For angiography, radiopaque Microfil orange (Canton Biomedical Products, Boulder, CO) was used to fill the complete vasculature and after 3 h was cured to a solid. The whole mouse or sliced tumor (2-3 mm thick) was then x-rayed using a Faxitron apparatus. The parameters for optimal x-ray contrast from the Faxitron were determined to be 45 kV for 7.5 minutes using Kodak Industrex R Ultrafine grain, high contrast x-ray film.

For LM, sample tumors were resected, fixed in formalin by immersion in order to leave the red blood cells in place as markers, sectioned and stained with either hematoxylin-eosin or Masson's trichrome stain for microscopic examination. Stereological point counting was used to determine the volume of tissue occupied by the blood cells or intravascular space.

For casting, the two components of undiluted Mercox (Ladd Research Industries, Burlington, VT) were mixed in a 10 ml syringe and immediately injected through the fixation cannula. Because of prefixation of the dilated vasculature the injection pressure was not controlled, but sufficient pressure for the viscosity of 20-30 cp was used to fill the vasculature completely. Reducing the amount of catalyst increased the working time, which permits the infusion of several mice. The next day the entire animal was placed in a 30% NaOH solution at 55°C to digest the fixed tissue. The NaOH solution was changed daily for a few days until only the cast of the mouse vasculature remained. The cast was then rinsed with distilled water and allowed to dry in a desiccator. The shrinkage of Mercox casts is approximately 8% (Weiger et al., 1986).

For SEM, the casts were mounted with double-sided-gluing tape and grounded with wet carbon. They were sputter coated with gold-palladium to a thickness of 20nm. The accelerating voltages used were between 5 and 15 kV but 10 kV was usually employed. The stage was often tilted and appropriate electronic compensation was used to preserve accurate dimensions. Polaroid positive prints and negatives were used to produce the figures below. The TV mode of the SEM (Cambridge Stereoscan 100T) was used in conjunction with a Beta VCR and microcomputer in order to measure digitized lumenal dimensions of digitized images.

For TEM, the tumors were resected immediately after euthanasia or after perfusion fixation as described above. Small (2mm) cubes of tissue were excised from the tumors and immersed in 2% glutaraldehyde, 1.25% paraformaldehyde, 0.09% $CaCl_2$ in 0.1 M cacodylate buffer with sucrose. The pieces of tissue were taken from well defined locations varying from the outside to the inside of the tumor and prepared for TEM using standard procedures.

Results

As suggested by Lametschwandtner et al. (1984), an attempt was made to determine which vessels were feeding the tumor by using Microfil. This has served two purposes, 1) to make sure that the injection site for casting is adequate for filling all vessels associated with the tumor and 2) to determine which major vessels of the body were associated with the origin vessels for supplying or draining of blood from the tumor. In preference to usual methods of clearing, x-rays were taken of the whole mouse such that the radiopaque Microfil highlighted the vasculature perfused from the aortic injection site (Fig. 1). Note in Fig. 1 that the tumor in the pelvic region is supplied by at least one long blood vessel (most likely a vein) extending from the apical blood vessels. The x-rays of the in situ tumor in Fig. 1 and isolated tumor slice in Fig. 2 show pooled areas of Microfil corresponding to extravascular efflux. The observed spilling into the central area of tumors is characteristic of these tumors as they start developing a necrotic core. Such tumors correspond in size to those of the exponential growth phase in young rats as indicated in Fig. 3. In order to avoid the problem of the extravascular efflux in this investigation, smaller tumors were studied. Tumors were grown for approximately 14 days in young hosts and 21 days in old hosts resulting in a final tumor volume of approximately 250 mm^3. All of the results described below correspond to tumors of this size.

In order to obtain the data for Fig. 4, photomicrographic montages were made through the midsection of histological sections of a young- and old-host tumor. A grid was superimposed on the micrographs at 1/2 mm intervals and the points intersecting blood or vascular space were counted and divided by the total

Fig. 1. Print of x-ray of young mouse with a B16 melanoma tumor in the pelvic region 21 days after inoculation. The radiopaque Microfil appears as dark in vascular and extravascular space. A blood vessel feeds the tumor from an apical origin (between arrows corresponding to a distance of 3.4 cm). There are upper (U) and lower (L) lobes for the tumor.

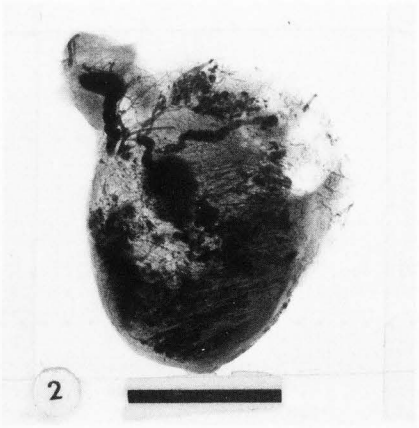


Fig. 2. Print of x-ray of tumor section (2-3 mm thick) taken from a young mouse 21 days after inoculation. This micro-angiogram vessel radiates inwards and there is some extravascular leakage into the central necrotic region. Bar = 1 cm.

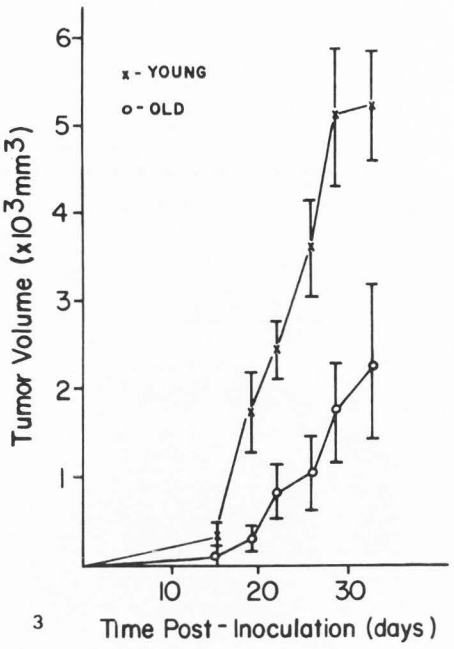


Fig. 3. Tumor growth in young and old mice (N ≥ 5 for each group). The tumor size was measured in two dimensions and the tumor volume ± SE was calculated as described by Ershler et al. (1984a).

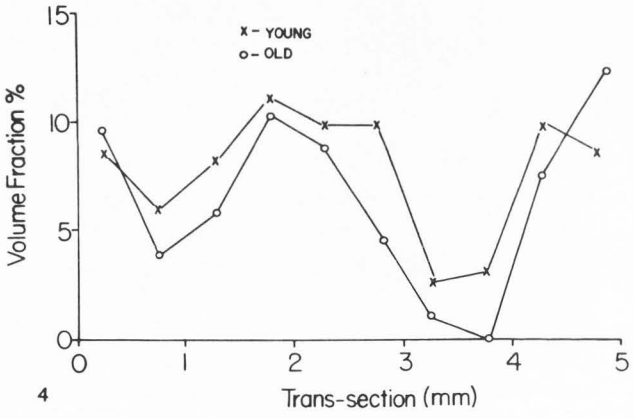


Fig. 4. Blood or vascular volume variation through the midsection of typical ≈250 mm³ tumors (comparable in size to those in Figs. 5 and 6). The trans-section distance is from the lateral margin of tumor (0) to the medial margin (5 mm).

number of points intersecting the tissue to give the volume fraction. Note that except for the extreme edges of the tumor there is uniformly less blood supply to the tumor of the old mouse. This difference between young and old is consistent with the SEM observations described below. The presence or absence of vascular wall around erythrocytes was determined using the light microscope directly. In the central region of the tumors the vascular wall was poorly defined or absent. This characteristic avascularity was more common for old rather than young hosts.

The overall SEM views of the vascular corrosion casts from the young (Fig. 5) and the old (Fig. 6) mice illustrate the profound difference in blood supply to the ≈250 mm³ tumors. The margin of the tumor from the young mouse is obvious (Fig 5) while only the left margin of the tumor from the old (Fig. 6) seems to be delimited by the microvascular network. Note that for the old there seems to be no lack of potential feeding vessels surrounding the open dark space in the middle formerly occupied by the tumor tissue (Fig. 6). The lumen of the large feeding vessel at the top of the young-host tumor is straight and of uniform diameter while for the old-host the lumens are convoluted and more variable in diameter.

At the margin of the tumor there appear to be vessels which extend from the larger feeding vessels and either penetrate or attempt to penetrate into the tumor space (Figs. 7 and 8). For the young mice, the vessels appear to have a regularly shaped tapering lumen penetrating into the tumor and giving off numerous buds (Fig. 7). For the old mice, the vessels are often very convoluted, abruptly taper off and do not give rise to many sprouts (Fig. 8). it can be questioned whether these vessels successfully penetrate into the tumor and if they do not what prevents such penetration.

As the penetrating vessels taper off they can give rise to a dense vascular network characterized by many collaterals rather than an ordered branching hierarchy (Figs. 9 and 10). Because of their location at the edges of the tumor they can be called peripheral or marginal vessels. For young hosts, the bulbous and invaginated nature of the lumens is consistent with neoangiogenesis and expansion of the vascular volume (Fig. 9). For old hosts, the twisted string-like nature of the lumens is more characteristic of a mature microcirculatory bed except that there is a sparse and disproportionate distribution of the vessels (Fig. 10).

In order to detect whether there is likely to be angiogenesis near the avascular zone in the central part of the tumor, the lumenal cast of the innermost vessels was observed (Figs. 11 and 12). These views were accomplished by tilting the stage and brushing away peripheral vessels where necessary. For the young mice, the lumens of these vessels often have fungiform bulges, with holes and furrows characteristic of neoangiogenesis (Fig. 11). For old mice, the inner vessel casts have similar characteristics to those of the peripheral vessels almost devoid of any bulges or sprouting (Fig. 12).

The TEM of tumor vessels simply illustrates that the wall structure of these vessels is different from that of the healthy vascular network whether the tumors are in old or young hosts. The only normal feature of the ≈70 μm diameter tumor vessel in Fig. 13 is the intact endothelium. The basement membrane varies in density and thickness around the circumference and there are no typical smooth muscle cells. A normal vessel of this size would have both a regular basement membrane and smooth muscle

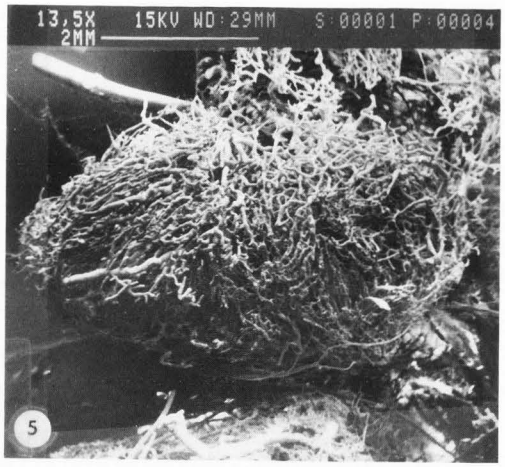


Fig. 5. SEM of vascular corrosion cast of tumor from a young mouse 14 days after inoculation. Note that the distance bar in mm is indicated at the top of the figure as it appeared on the CRT of the SEM. For some of the following micrographs the bar lengths are given in the caption. In addition to the bar the display also indicates the magnification, accelerating voltage, working distance, specimen number and photo number. All SEM views for the young host as shown below are from this cast but are characteristic of other casts for this size and age (odd-numbered figures).

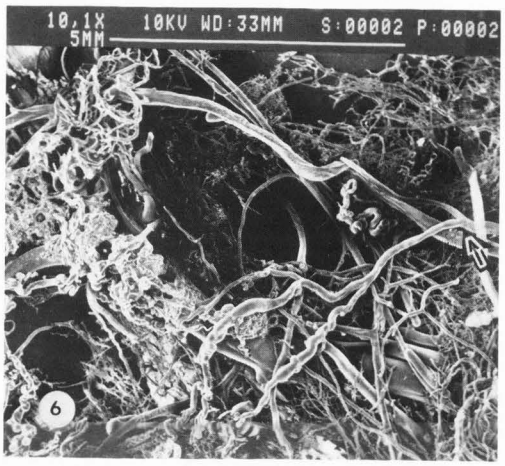


Fig. 6. SEM of vascular corrosion cast of tumor from an old mouse 21 days after inoculation. Before digestion, the original tumor tissue extended from the arterial bifurcation at the right (arrow) to just beyond the crescent-shaped microvascular network at the left. All SEM views for the old host as shown below are from this cast but are characteristic of other casts for this size and age (even-numbered figures).

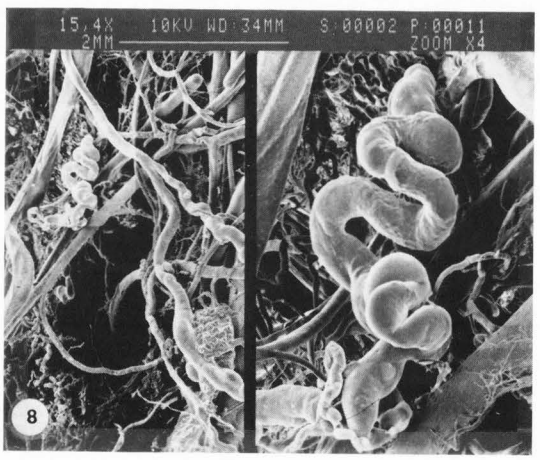


Fig. 8. Old-host feeding or penetrating vessel lumens. The right frame is a 4X zoom of the frame at the left. The bar for the left is 2 mm as indicated while for the right this bar represents a length of 500 μm. The three-dimensional convolution of the vessel on the right is obvious. Its origin was at the arterial bifurcation on the margin of the tumor space and there is very little microvascular structure extending from its lower end.

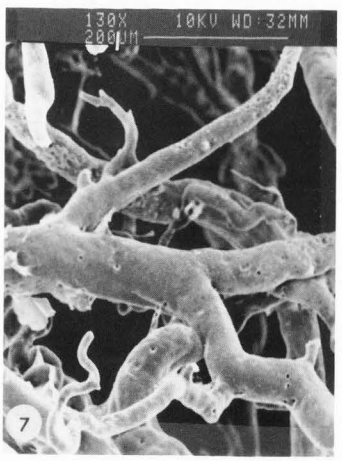
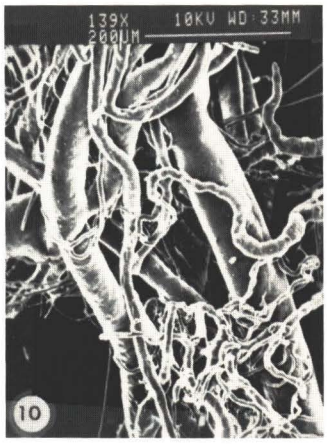
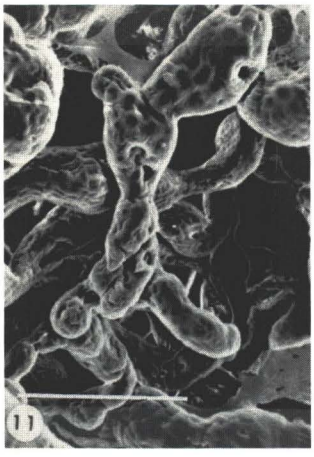
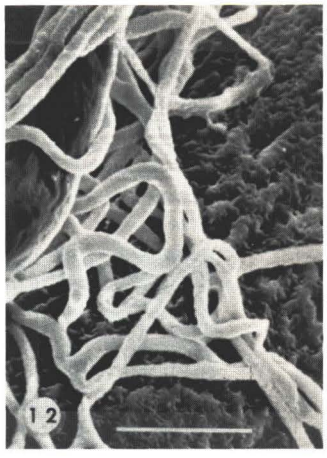


Fig. 7. Young-host feeding or penetrating vessel lumens. The prominent vessel gives rise to fairly straight branches which taper gradually and give off vascular sprouts of varying length.

layer. As for the capillary like vessels, they have loose intercellular junctions as indicated by cellular protrusions into the lumen (Fig. 14).







Discussion

Tumor growth requires local host factors that might be deficient in the old animals. If the adjacent host cells do not respond to the tumor cell-producing tissue angiogenesis factor, then the resulting lack of neovascularization may inhibit tumor growth (Folkman, 1986). Our SEM casts and other results which have demonstrated less vascularity in the tumors from the older hosts, support this contention. Additionally, fibrogenic factors may offer explanations as well. Ershler et al. (1984a; 1984b) attempted to explain the apparent age advantage in retarding tumor growth by proposing the presence of a greater fibrogenic component resulting in a containment mechanism which was more successful in the senescent host. Alternatively, the fibrosis of the tumor could prevent the penetration of the feeding vessels into the tumor tissue as suggested by the convoluted vessel in Fig. 8. We did not see this type of tortuosity in penetrating vessels of young mice (Fig. 7), but Grunt et al. (1986a, Diagram 6) describe this characteristic convolution in their tumor model. Nearly every other feature described by us for young hosts has also been described by Grunt et al. (1986a; 1986b).

Fig. 9. Young-host peripheral vessel cast.

Fig. 10. Old-host peripheral vessel cast.

Fig. 11. Young-host inner-vessel cast near avascular zone. Bar = 50 μm.

Fig. 12. Old-host inner-vessel cast near avascular zone. Bar = 50 μm.

Fig. 13. TEM. A feeding blood vessel in tumor from a young mouse. A prominent endothelial nucleus is indicated with short arrow. The cell marked with long arrow may be associated with the developing vascular wall (pericyte or smooth muscle). Bar = 10 μm.

Fig. 14. TEM. A developing blood vessel or capillary in tumor taken from a young mouse. Lumen is practically filled by an erythrocyte. An intercellular junction is associated with cellular protrusion into the lumen (arrow). Bar = 2 μm.

Their overall diagrams of the supply and drainage vessels which we call feeding vessels are similar to the observations made by us with angiography (Fig. 1) and SEM of casts (Fig. 5). Their description of a "peripheral vascular envelope" and "giant capillaries" in terms of bulges, holes and folds agree well with our observations (Figs. 9 and 11). These features are characteristic of neoangiogenesis which is apparent in tumors of young hosts. In conclusion, it is possible to compare the vasculature of similar sized tumors of young and old animals. The B16 melanoma tumors of old mice have more necrosis, fewer angiogenic features, decreased vessel density, reduced vessel penetration into the tumor, and enhanced tortuosity of vessels. Transmission electron microscopy revealed incompletely developed wall structure of the vessels regardless of host (Figs. 13 and 14). The above results are consistent with the hypothesis that retarded angiogenesis is characteristic of slower growing tumors in senescent animals and may be primarily responsible for restricting growth while being modified by other factors.

Acknowledgements

We are grateful for the technical and editorial assistance of R.M. Gundel and P.J. Kimberly. The B16 melanoma was provided by Dr. I. Fidler. Supported by American Cancer Society Grant #IN156A, USPHS Grants K08 AG 00214 and R23 HL32334.

References

Altman PL, Katz DD. (1979). Inbred and genetically defined strains of laboratory animals. Part I. Mouse and rat. Bethesda, MD FASEB, 45-50.

Ershler WB, Socinski MA, Green CJ (1983). Bronchogenic cancer, metastases and aging. J. Amer. Geriatrics Soc. 31, 673-676.

Ershler WB, Stewart JA, Hacker MP, Moore AL, Tindle BH (1984a). B16 murine melanoma and aging. Slower growth and longer survival in old mice. J. Nat. Can. Inst. 72, 161-164.

Ershler WB, Gamelli RL, Moore AL, Hacker MP, Blow AJ (1984b). Experimental tumor and aging: Local factors that may account for the observed age advantage in the B16 murine melanoma model. Experimental Gerontology 19, 367-376.

Folkman, J (1986). How is blood vessel growth regulated in normal and neoplastic tissue? -- G.H.A. Clowes memorial award lecture. Cancer Res. 46, 467-473.

Gatti RA, Good RA (1970). Aging immunity and malignancy. Geriatrics 25, 158.

Grunt TW, Lametschwandtner A, Karrer K, Staindl O (1986a). The angioarchitecture of the Lewis lung carcinoma in laboratory mice. A light microscopic and scanning electron microscopic study. Scanning Electron Microsc. 1986; II: 557-573.

Grunt TW, Lametschwandtner A, Karrer K (1986b). The characteristic structural features of the blood vessels of the Lewis lung carcinoma. A light microscopic and scanning electron microscopic study. Scanning Electron Microsc. 1986; II: 575-583.

Lametschwandtner A, Lametschwandtner U, Weiger T (1984). Scanning electron microscopy of vascular corrosion casts - technique and applications. Scanning Electron Microsc. 1984; II: 663-695.

Miller DG (1980). On the nature of susceptibility to cancer. Cancer 46, 1307-1318.

Peto R, Roe FJ, Lee PN, Levy L, Clack J (1975). Cancer and ageing in mice and men. Br. J. Cancer 32, 411-426.

Saitoh H, Shiramizu T, Hida M (1982). Age changes in metastatic patterns of renal adenocarcinoma. Cancer 50, 1646-1648.

Walford RL (1976). When is a mouse "old"? J. Immunol. 117, 352-353.

Warren BA (1966). The ultrastructure of capillary sprouts induced by melanoma transplants in the golden hamster. J. Royal Microsc. Soc. 86, 177-187.

Warren BA, Shubik P (1966). The growth of the blood supply to melanoma transplants in the hamster cheek pouch. Lab Invest. 15, 464-478.

Weiger T, Lametschwandtner A, Stockmayer P (1986). Technical parameters of plastics (Mercox CL-2B and various methymethacrylates) used in scanning electron microscopy of vascular corrosion casts. Scanning Electron Microsc. 1986; I: 243-252.

Young JL, Asire AJ, Pollack ES (1976). SEER Program: Cancer incidence and mortality in the U.S., 1973-1976. U.S. Department of Health, Education and Welfare, National Institutes of Health 78:1837, Washington, D.C. U.S. Government Printing Office.

Discussion With Reviewers

R.M. Albrecht: Have the authors attempted casting of unfixed vessels and, if so, do the results vary from those obtained with prefixed material?

Authors: Yes, we have attempted to use Mercox for casting of the unfixed vasculature. Resultant casts were characterized by more segmental variability in the diameter of large vessels and vastly reduced filling of smaller blood vessels. Both procedures involved pre-

liminary flushing of the vasculature with the vasodilator and heparin (see Methods), the poor results using direct Mercox injection may be explained by increased pressure drop along the vascular network due to the higher viscosity of the Mercox.

R.M. Albrecht: The procedures section mentions the use of stained sections to determine the volume of vasculature. Did the authors notice significant fibrosis at the point where the vasculature decreases sharply, in other words did the fibrosis correlate directly with the vessel distribution?
Authors: The Masson's trichrome stain used in conjunction with light microscopy highlighted acellular components which seemed to increase in proportion as the vasculature decreased sharply. However, both the qualitative description and quantitative comparison of fibers within the acellular component would have to be analyzed using transmission electron microscopy in a detailed systematic manner.

R.M. Albrecht: From the casting results there appears to be a major difference between the degree of internal vasculature in tumors grown in aged vs. young mice. The quantitative data presented in Fig. 4 seem to indicate a much smaller difference in the degree of vascularization. Could the authors comment?
Authors: The apparent difference in views of casts and the proportional composition measurements of Fig. 4 result from the fact that different vascular compartments are being measured. Presumably, the cast remains well formed and continuous only when it is contained inside vessels with intact walls. All blood containing tissue is counted for Fig. 4 which would include rudimentary leaky vessels and blood pools.

R.M. Albrecht: Based on the methodology described, it is not clear why the point of minimal vascularity, in both the tumors from young and old mice, lies on one side, the same side of center (Fig. 4). Are the tumors oriented in some way prior to measurement?
Authors: Yes, the tumors are oriented in the same way with 0 trans-section corresponding to the lateral edge of the tumor and the opposite edge corresponding to the medial. This would be consistent with the view in Fig. 1 with an increase in feeding vessel caliber on the lateral or peritoneal side.

R.M. Albrecht: Are the same amounts and types of vessel growth factors produced when this tumor type grows in old as opposed to young mice?
Authors: From our current understanding (Folkman, 1986), both vessel growth and inhibitory factors exist in malignant and normal tissues. Growth factors stimulate host capillary endothelial motility, invasiveness, and proliferation. As your question suggests, it is possible that angiogenesis factors elicit different responses in different tissue sites and in different hosts (e.g. young and old). The results of this paper illustrate the importance of doing SEM and quantitative stereology in conjunction with measurement of vessel growth factors in this tumor line as well as others, but this has not yet been done.

B. Perksy: Is it possible that tumor volume in young and old mice merely reflects an animal's ability to inhibit the tumorigenic capability of B16 cells? Is there a relationship between the melanin characteristics of the tumor and the age of the mouse? Are there protocols in your materials and methods (e.g. cell preparation) that are known to alter tumorigenicity?
Authors: There are many age-related changes which may alter the tumorigenic capability of these cells other than angiogenesis. Among these changes are fibrotic response, immune senescence, endocrine senescence, and nutritional status of the host. Although the tumors grossly have the same melanin appearance, detailed studies have not been undertaken in this laboratory. As indicated in the Methods, cell viability was checked and the consistency of results implies that host age is the most important variable. Furthermore, similar differences in growth of Lewis lung carcinoma with host age have been observed in this laboratory.

K Hodde: You preferred not to use the usual clearing methods (Spalteholz) but rather use the radiopaque quality of Microfil for visualizing the vessels. I would suggest that after x-raying you could still clear the slices and observe the vessel morphological characteristics in transmitted LM, thus gaining additional information which should corroborate your SEM findings.
Authors: This is a very good suggestion and we have had some success clearing other tissues with standard techniques involving dehydration and clearing with methyl salicylate.

K. Hodde: Are the characteristics of blood vessels in the initial stage tumors similar in young and old mice and therefore comparable? Or, in other words, are changes in blood vessel morphology linked with the tumor stage in such a way that casting procedures do not work in both old and young hosts at all stages?
Authors: It certainly is worth pointing out that there are problems with corrosion casting in larger tumors. Perhaps because of excessive leaking, there are often pools of the casting material which can cause the casts to fall apart upon corrosion. Comparisons at later stages of tumor growth then become more difficult because of variable and irregular casts. Also as can be inferred from Fig. 3, tumors in older mice never get to the largest size found in younger mice. Size is probably the best criterion for determining the tumor stage, which is possible but subject to the limitations of the casting technique described above. However, if the cast is intact and the size has been determined it should be possible to predict whether the tumor was from an old or a young host.